PB81-126609

Effects of Adaptation on Biodegradation Rates in Sediment/Water Cores from Estuarine and Freshwater Environments

(U.S.) Environmental Research Lab.
Gulf Breeze, FL

Oct 80

U.S. Department of Commerce
National Technical Information Service
NTIS.

ERLGB J050

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. R PB81-126609
EPA-600/J-80-007	Journal Article	

4. TITLE AND SUBTITLE	5. REPORT DATE
Effects of Adaptation on Biodegradation Rates in Sediment/Water Cores From Estuarine and Freshwater Environments.	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Jim C. Spain, P. H. Pritchard, and A. W. Bourquin	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
SAME	A1VL1A
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Environmental Research Laboratory Office of Research and Development Environmental Protection Agency Gulf Breeze, FL 32561	14. SPONSORING AGENCY CODE ORD/600/4

15. SUPPLEMENTARY NOTES

REF: Applied and Environmental Microbiology, October 1980, pp. 726-734

16. ABSTRACT

Experiments were devised to determine whether exposure to xenobiotics would cause microbial populations to degrade the compounds more rapidly during subsequent exposures. Studies were done with water/sediment systems (eco-cores) taken from a salt marsh and a river. Systems were tested for adaptation to the model compounds methyl parathion and *p*-nitrophenol. $^{14}CO_2$ released from radioactive parent compounds was used as a measure of mineralization. River populations pre-exposed to *p*-nitrophenol at concentrations as low as 60 µg/l degraded the nitrophenol much faster than control populations. River populations pre-exposed to methyl parathion also adapted to degrade the pesticides more rapidly, but higher concentrations were required. Salt marsh populations did not adapt to degrade methyl parathion. *p*-Nitrophenol-degrading bacteria were isolated from river samples, but not from salt marsh samples. Numbers of nitrophenol-degrading bacteria increased 4 to 5 orders of magnitude during adaptation. Results indicate that the ability of populations to adapt depends on the presence of specific microorganisms. Biodegradation rates in laboratory systems can be affected by concentration and prior expsoure; therefore, adaptation must be considered when such systems are used to predict the fate of xenobiotics in the environment.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Fate	Eco-core	06/F
Biodegradation	Methyl-parathion	06/M
Adaptation	*p*-nitrophenol	06/T
Mineralization		06/S

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	Unclassified	
	20. SECURITY CLASS (This page) Unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA-600/J-80-007
Journal ARticle

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1980, p. 726–734 Vol. 40, No. 4
0099-2240/80/10-0726/09$02.00/0

Effects of Adaptation on Biodegradation Rates in Sediment/Water Cores from Estuarine and Freshwater Environments†

JIM C. SPAIN,‡* P. H. PRITCHARD, AND A. W. BOURQUIN

United States Environmental Protection Agency, Environmental Research Laboratory, Gulf Breeze, Florida 32561

Experiments were devised to determine whether exposure to xenobiotics would cause microbial populations to degrade the compounds more rapidly during subsequent exposures. Studies were done with water/sediment systems (ecocores) taken from a salt marsh and a river. Systems were tested for adaptation to the model compounds methyl parathion and *p*-nitrophenol. $^{14}CO_2$ released from radioactive parent compounds was used as a measure of mineralization. River populations preexposed to *p*-nitrophenol at concentrations as low as 60 μg/liter degraded the nitrophenol much faster than did control populations. River populations preexposed to methyl parathion also adapted to degrade the pesticide more rapidly, but higher concentrations were required. Salt marsh populations did not adapt to degrade methyl parathion. *p*-Nitrophenol-degrading bacteria were isolated from river samples but not from salt marsh samples. Numbers of nitrophenol-degrading bacteria increased 4 to 5 orders of magnitude during adaptation. Results indicate that the ability of populations to adapt depends on the presence of specific microorganisms. Biodegradation rates in laboratory systems can be affected by concentration and prior exposure; therefore, adaptation must be considered when such systems are used to predict the fate of xenobiotics in the environment.

Microbial degradation of organic compounds is often investigated in the laboratory by using mixed culture systems obtained from the environment (1, 2, 8, 9). Frequently, such investigations are designed to measure biodegradation rate constants that can be used to predict potential biodegradation rates in the environment. It is difficult, however, to extrapolate results obtained in laboratory systems to predict the fate of organic pollutants in the environment, where conditions may be different from those in the laboratory. The effects of many parameters must be considered: temperature, salinity, pH, oxygen concentration, nutrient concentration, inoculum size, source of inoculum, chemical structure and concentration of the test compound, and adaptation of the microbial population to degrade the test compound. The effects of adaptation are probably the least defined.

Microbial populations can often adapt to transform specific compounds more rapidly. For example, Haller (6) has studied adaptation of sewage populations to degrade several aromatic compounds, and Hollibaugh's recent studies (7) clearly show that natural bacterial populations can adapt to metabolize certain amino acids.

There are three ways by which adaptation can occur upon exposure of the population to a new substrate: (i) induction or derepression of specific enzymes not present (or present at low levels) in the population before exposure, (ii) selection of new metabolic capabilities produced by genetic changes, and (iii) increase in the number or organisms able to catalyze a particular transformation. The third type of change often follows one of the first two. Any of the above types of adaptation may drastically change the biodegradation rate constants observed in laboratory test systems. Rate constants determined by using adapted populations (8) may not be comparable with those determined by using unadapted populations (1). Substrate concentration may determine whether populations can adapt, i.e., there may be concentration thresholds below which no adaptation takes place (2). Adaptation may also be affected by the type and amount of inoculum, the structure of the test compound, physical parameters, or the design of the test system. The purpose of this paper is to describe our investigations of adaptation in natural populations exposed to the synthetic organic compounds methyl parathion (MP) and *p*-nitrophenol (PNP). The major objective of this work was to determine whether adaptation could affect predictions of biodegra-

† Contribution no. 410 from the Gulf Breeze Laboratory.

‡ Permanent address: University of Texas, Austin, TX 78712.

dation rates for xenobiotics at low concentrations in the environment.

MATERIALS AND METHODS

Test system. Biodegradation experiments were done with mixed microbial populations obtained from natural environments. The "eco-core" test system described by Pritchard et al. (9) served as both a sampling device and a container during incubation. The system consisted of a glass tube (3.5 by 40 cm) sealed at each end with silicone stoppers. An 18-gauge hypodermic needle 20 cm long (Becton, Dickinson & Co., Rutherford, N.J.) was inserted through the upper stopper for the introduction of air into the system during incubation. A short length of 9-mm glass tubing inserted through the upper stopper provided an outlet for effluent gases.

For filling the sterile eco-cores with test material, the lower stopper was removed at the sampling site; the tube was inserted vertically into the sediment to a depth of 78 cm; the core was carefully removed from the sediment, retaining a sediment plug in the bottom of the tube; and the stopper was replaced. Since the water depth at all sampling sites was greater than 50 cm, the space in the tube above the sediment filled with water during the procedure. Samples were transported to the laboratory as soon as possible, and water volumes were adjusted to 175 ml per core. For degradation studies, the cores were incubated at 25°C unless otherwise indicated; air was gently introduced into the water column 3.0 cm above the sediment via the submerged hypodermic needle and effluent gases were passed through a solution of 1.0 N sodium hydroxide to remove carbon dioxide. Mineralization rates of radioactive test substrates were determined by measuring the amount of radioactivity associated with the trapped carbon dioxide. The trapped radioactivity was released when samples of the traps were acidified to pH 2.0, thus confirming that the radioactivity was associated with CO_2.

In adaptation experiments in which the degradation rate of a compound was to be studied after a second exposure of the microbial test system to the compound, the water in the system was changed before the second spike to prevent isotope dilution (Fig. 1). To accomplish the change, the water layer was transferred to sterile centrifuge bottles; particulate material was collected by centrifugation and the supernatant fluid was discarded. The particulate material was then suspended in 175 ml of water from the original sampling site that had been sterilized by passage through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). Suspensions thus prepared were returned to the cores with minimal disruption of the sediment layer and labeled test compounds were added.

Extraction and analysis of PNP and MP. Water samples were extracted twice at neutral pH with equal volumes of ethyl acetate, acidified to pH 1.5 with HCl, and extracted twice more. The ethyl acetate was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The resultant residues were dissolved in acetone and stored at −20°C until analyzed.

For thin-layer chromatographic analysis, extracts were applied to glass plates precoated with silica gel (LK6DF; Whatman Ltd., Clifton, N.J.). Chromatograms were developed with chloroform-acetone (80:20). Compounds were detected on the chromatograms by fluorescence quenching when viewed under ultraviolet light (254 nm). Identification was accomplished by comparison with authentic standards. Radioactive areas were detected on the plates with a spark chamber (Birchover Instruments Ltd., Letchworth, Herts, England). Radioactive compounds were extracted from the plates, and the radioactivity was measured by liquid scintillation counting.

Culture media and conditions. Basal growth medium was prepared from a mineral salts base (MSB) which contained, per liter: K_2HPO_4, 700 mg; $MgSO_4 \cdot H_2O$, 112 mg; $ZnSO_4$, 5 mg; $NaMoO_4 \cdot 2H_2O$, 2 mg; $CaCl_2$, 14 mg; and NH_4Cl, 500 mg. The basal medium was supplemented with PNP or MP at a concentration of 50 mg/liter and yeast extract at 50 mg/liter for enumeration of bacteria able to degrade the test compounds. The pH of the medium was adjusted to 7.4. Sodium chloride was added to the medium to a concentration of 1.5% for growth of bacteria from the salt marsh sampling site. Solid media were prepared by adding agar (Difco Laboratories, Detroit, Mich.) to the liquid media at a final concentration of 15 g/liter before autoclaving. For enumeration of total heterotrophic bacteria, the minimal medium was supplemented with Difco nutrient broth (2 g/liter).

Enumeration, selection, and isolation of bacteria. Enumeration of bacteria from the eco-cores was accomplished by inverting the cores several times to resuspend the detritus layer (upper 1 to 2 cm) of the sediment in the overlying water; samples of the result-

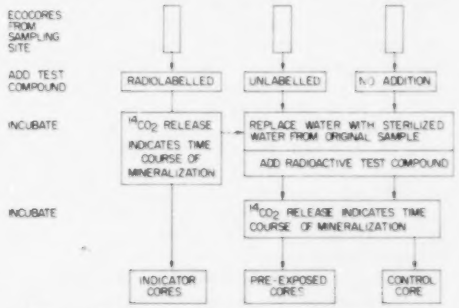

FIG. 1. *Flow diagram for experiments used to investigate adaptation of microbial populations after exposure to organic substrates. Cores taken at the sampling site were treated in one of three ways. One group of cores was spiked immediately with ^{14}C-labeled test compound (indicator). A second group was spiked with unlabeled test compound (preexposed). The third group received no addition (control). $^{14}CO_2$ release from the indicator cores was followed as described in the text. When the rate increased substantially, the preexposed and control cores were spiked with radiolabeled test material, and $^{14}CO_2$ release was followed as a measure of mineralization. A substantially higher rate in preexposed systems was taken to be evidence of adaptation.*

ant suspension were diluted and spread on the surface of agar plates containing the appropriate carbon source. The plates were incubated for 5 days at 25°C, and colony-forming units were determined.

Bacteria were isolated by selective enrichment in growth medium containing either MP or PNP. The sediment in eco-cores was suspended in the overlying water as described above. Samples of the suspension were diluted 10-fold with artificial growth medium containing either PNP or MP, and the suspensions were incubated at 25°C with shaking in flasks of the appropriate size. At intervals, samples of the suspension were removed, and the amount of PNP or MP remaining was determined by extraction and thin-layer chromatographic analysis. When the test compound disappeared from the medium, samples of the suspension were removed, diluted 10-fold with fresh medium, and incubated as before. When the MP or PNP disappeared the second time, samples of the suspension were spread on the surface of agar plates containing the appropriate substrate. After incubation for 72 h at 25°C, material from the larger colonies was transferred to fresh agar medium and further incubated. After several transfers, organisms that continued to grow well were tested for the ability to degrade PNP or MP as follows. Bacteria were grown overnight in liquid medium supplemented with MP or PNP; radiolabeled MP or PNP (100 μg/liter final concentration) was added to the culture suspension, and the suspension was incubated at 25°C with shaking for 48 h; samples of the suspension were removed and examined for the presence of MP or PNP by extraction and thin-layer chromatographic analysis. Disappearance of the parent compound was taken to indicate degradation. The concentration of the compounds remained stable during the incubation period when cell suspensions were treated with Formalin (2%, vol/vol) before incubation. PNP degradation was detected by following the disappearance of yellow color from the medium during growth of the bacteria.

Materials. PNP was obtained from Matheson, Coleman and Bell, Inc. (Norwood, Ohio); MP (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothioate) was from Monsanto Co. (St. Louis, Mo.). [*U*-^{14}C]PNP and [*ring*-*U*-^{14}C]MP were from Pathfinder Laboratories Inc. (St. Louis, Mo.). Purity of chemicals was verified by thin-layer chromatographic analysis before use.

Radioactivity measurements. Material in the CO_2 traps was prepared for counting by dissolving a 1.0-ml sample from the trap in Carbon 14 cocktail (R. J. Harvey Instrument Co., Hillsdale, N.J.). PCS liquid scintillation cocktail (Amersham, Arlington Heights, Ill.) was used for other types of samples. Radioactivity was measured by liquid scintillation with a Beckman LS250 liquid scintillation counter. All samples were corrected for background and quenching.

Sampling sites. Sediment and water samples used in the study were obtained from two different aquatic environments. The estuarine sampling site was at Range Point, a salt marsh adjacent to Santa Rosa Sound near Pensacola Beach, Florida. When the samples were taken salinity was 1.3%, water temperature was 26°C, and water depth was 60 cm. The sediment consisted of a thin (<0.5-cm) layer of detritus overlying sand.

The freshwater sampling site was in the Escambia River 8 miles above Escambia Bay and upriver from known industrial effluent discharges in the Pensacola area. Water depth at the sampling site was 1.5 m, and the temperature was 24°C. The sediment contained a thick layer of organic material and released a considerable amount of gas when disturbed.

RESULTS

Preliminary adaptation experiments. In a preliminary experiment to determine whether adaptation could be detected in mixed cultures by following mineralization, a number of eco-cores were taken from the Escambia River sampling site on 13 August 1979, spiked with radiolabeled MP or PNP, and treated as shown in Fig. 1. Each compound was added at two different concentrations; $^{14}CO_2$ released from cores during subsequent incubation was followed as an indicator of parent compound mineralization (Fig. 2). The rate of $^{14}CO_2$ release increased sharply after 60 h in cores containing the higher concentrations of both MP and PNP. The rate of $^{14}CO_2$ release from cores containing lower concentrations of the two compounds did not change abruptly during the experiment. The abrupt increase in the rate of $^{14}CO_2$ release from cores spiked at the higher concentrations suggested that some type of adaptation had occurred.

For more careful investigation of the occurrence of adaptation, similar cores (taken at the same time as those described above) were spiked initially with unlabeled MP or PNP as outlined in Fig. 1. Each compound was added to several cores at a concentration of 0.45 μM and to several others at a concentration of 180 μM. Each

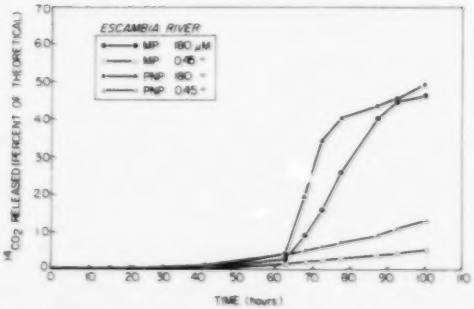

FIG. 2. *Mineralization in Escambia River eco-core samples. Cores taken 13 August 1979 were supplemented immediately with [^{14}C]MP or [^{14}C]PNP and then incubated with aeration at 25°C. $^{14}CO_2$ released from the cores was trapped, and the radioactivity was measured as described in the text. Each compound was tested at concentrations of 0.45 and 180 μM.*

treated core was matched with a duplicate that did not receive MP or PNP. After incubation for 100 h (the appropriate time was determined from the results shown in Fig. 2), the water was removed from the cores as described above and replaced with filter-sterilized Escambia River water. Cores thus treated were then spiked with radiolabeled MP or PNP at the same concentration as the initial spike, and $^{14}CO_2$ release was followed (Fig. 3). $^{14}CO_2$ was released rapidly and without a lag from cores that were preexposed to 180 μM PNP. The rate of $^{14}CO_2$ release from control cores (no preexposure) did not become significant until 50 h after the spike. Cores preexposed to MP (180 μM) and respiked with radiolabeled MP released $^{14}CO_2$ much more rapidly than did control cores.

Cores that received lower concentrations (0.45 μM) of the parent compounds (Fig. 3B) released $^{14}CO_2$ at a much lower overall rate. However, cores preexposed to PNP were again much more active than were control cores. Preexposure of cores to MP at the lower concentration had little effect on the rate of subsequent MP mineralization.

The results indicate that both MP and PNP were mineralized much more rapidly in cores that were preexposed to the compounds at a concentration of 180 μM. Only PNP produced a detectable adaptive response at the lower concentration. Comparison of mineralization rates in preexposed and control cores provided a means to clearly establish whether the populations were adapted.

The preceding experiment was repeated with eco-cores taken from Range Point salt marsh on 27 August 1979. $^{14}CO_2$ release from cores spiked immediately with labeled MP or PNP is shown in Fig. 4. The rate of mineralization was lower in all cases than the rates in similarly treated cores from the Escambia River. It was not readily apparent from the plots of $^{14}CO_2$ release after the initial spike whether adaptation took place. The rate of release began to increase in the core that contained the higher concentration of PNP after about 140 h. However, the increase was not nearly as abrupt as that seen in Escambia River cores (Fig. 2).

Similar Range Point cores were preexposed to unlabeled MP or PNP as described in Fig. 1 and respiked with labeled compounds after 250 h of preexposure. Figure 5 shows that $^{14}CO_2$ was released much more slowly from Range Point cores than from Escambia River cores used in earlier experiments (Fig. 3). The rate was similar to that shown by cores initially spiked with

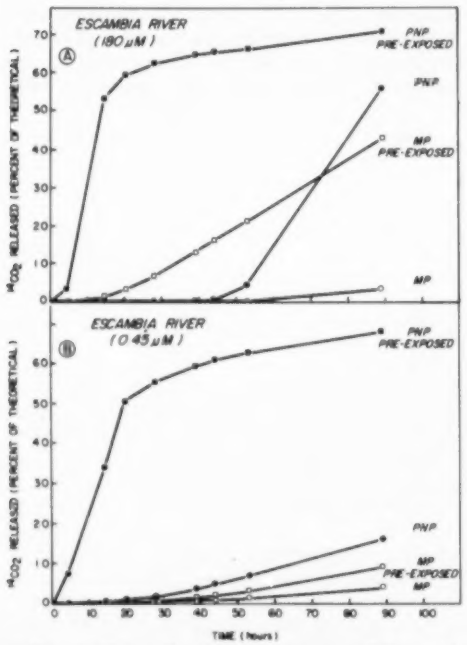

FIG. 3. *Mineralization in preexposed Escambia River cores. Eco-cores were taken at the Escambia River site and spiked immediately with unlabeled MP or PNP at concentrations of 0.45 and 180 μM. Control cores received no supplement. Cores were incubated 100 h, water was removed and replaced with filter-sterilized river water, and cores were respiked with [^{14}C]MP or [^{14}C]PNP at a concentration of 180 μM (A) or 0.45 μM (B). $^{14}CO_2$ release was followed during subsequent incubation.*

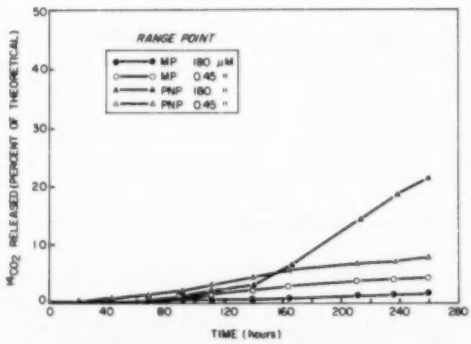

FIG. 4. *Mineralization in Range Point eco-cores. Eco-cores were taken at the Range Point salt marsh site. Cores were spiked immediately with [^{14}C]MP or [^{14}C]PNP and then incubated with aeration at 25°C. $^{14}CO_2$ released from the cores was trapped, and the radioactivity was measured as described in the text. Each compound was tested at concentrations of 180 and 0.45 μM.*

radiolabeled compounds. Preexposure to the test compounds did not affect the rate of $^{14}CO_2$ release.

Substrate disappearance. The possibility existed that the increased rate of $^{14}CO_2$ evolution in preexposed cores did not reflect a parallel increase in the rate of parent compound disappearance. To relate $^{14}CO_2$ release to [^{14}C]PNP disappearance, both were followed in cores taken from the Escambia River site on 10 October 1979 (Fig. 6). The pattern of $^{14}CO_2$ release in preexposed and control cores was similar to that in earlier experiments. In the preexposed core, PNP disappeared rapidly without a lag. In the control core, there was an initial, brief decrease in PNP concentration and then a lag, followed after 40 h by a relatively rapid decrease. The amount of radioactivity released as $^{14}CO_2$ was slightly less than the amount of PNP disappearance in both systems. The differences between preexposed and control cores were not as striking as in earlier experiments (Fig. 2), probably because the preexposure period was shorter, and adaptation may not have been complete. When the experiment was repeated with samples from Range Point, the nitro compound again disappeared slightly faster than $^{14}CO_2$ accumulated. The rate of substrate disappearance was no higher in preexposed cores than in control cores, which correlated well with the results obtained by measuring $^{14}CO_2$ release alone.

Effects of biomass. Since the differences in mineralization rates between cores taken from Range Point and from the Escambia River could be due to differences in microbial biomass at the two sites, numbers of heterotrophic bacteria were determined in sediment/water samples taken from each site in the experiments described in Fig. 2 to 5. Samples from the river contained 5.2×10^5 colony-forming units per ml; those from Range Point contained 8.0×10^4 colony-forming units per ml. The number of heterotrophs was thus 6.5 times higher at the river site, but the difference was not sufficient to explain the observed differences (up to 1,000-fold) in the mineralization rates.

Since differences in initial biomass at the two sites did not fully explain the disparity in mineralization rates, qualitative differences in the types of organisms at the two sites were investigated to determine whether there were indigenous organisms that were readily able to mineralize the test compounds. Samples from each site were tested for the presence of specific organisms able to degrade MP or PNP in pure culture. Selection techniques yielded five orga-

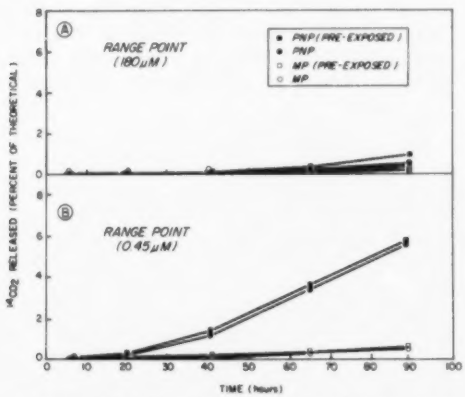

FIG. 5. *Mineralization in preexposed Range Point eco-cores. Cores were prepared at the Range Point sampling site and spiked immediately with unlabeled MP or PNP at concentrations of 180 μM (A) or 0.45 μM (B). Control cores received no supplement. Cores were incubated 250 h, water was removed and replaced with filtered Range Point water, preexposed and control cores were then respiked with [^{14}C]MP or [^{14}C]PNP at concentrations of 180 μM (A) or 0.45 μM (B), and $^{14}CO_2$ release was followed during subsequent incubation. Note the x-axis scale relative to that in Fig. 3.*

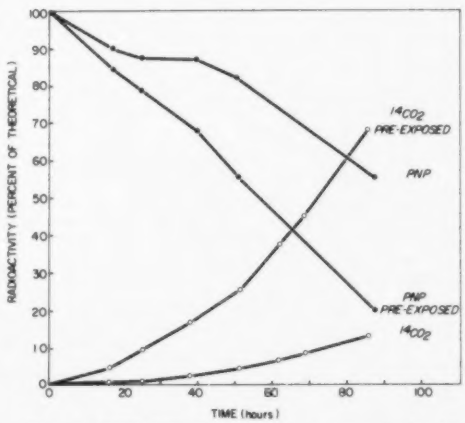

FIG. 6. *[^{14}C]PNP disappearance compared with $^{14}CO_2$ release in preexposed and control cores. Two eco-cores were prepared on 10 October 1979 at the Escambia River site and spiked immediately with PNP at a concentration of 0.72 μM. Two control cores received no supplement. Cores were incubated for 75 h, water was removed and replaced with filtered river water, and [^{14}C]PNP was added to a final concentration of 0.72 μM. $^{14}CO_2$ released from one preexposed and one control core was trapped, and the radioactivity was measured as described in the text. Water samples (5 ml) for extraction and analysis were removed from the duplicate cores at the indicated intervals. Extraction and quantitation of [^{14}C]PNP were performed as described in the text.*

TABLE 1. *Characteristics of organisms capable of PNP degradation*[a]

Isolate	Test response						Morphology	Genus
	Gram stain	Oxidase	Catalase	Motility	Nitrate reduction	Glucose fermentation		
B	−	+	+	+	+	−	Rod	*Pseudomonas*
D	−	+	+	+	+	−	Rod	*Pseudomonas*
1-U	−	+	+	+	+	−	Rod	*Pseudomonas*
2-P	−	−	+	−	+	−	Rod	*Acinetobacter*
1-Q	−	−	+	−	−	−	Rod	*Acinetobacter*
1-T	−	−	+	−	−	−	Rod	*Acinetobacter*

[a] Tests were done as described by Skerman (11); identification was done according to *Bergey's Manual of Determinative Bacteriology* (4).

nisms from Escambia River samples (Table 1) able to degrade PNP in pure culture, but none able to degrade MP. Attempts to isolate similar organisms from Range Point failed.

Results described thus far indicate that there was little or no adaptation in response to added MP or PNP in eco-core samples from Range Point. Moreover, the observed adaptation to MP by Escambia River samples could have been an adaptation to PNP released from MP by nonspecific hydrolysis. Therefore, to simplify the more detailed examination of adaptation, subsequent experiments were done with PNP as the model compound in eco-core samples from the Escambia River.

Population changes during adaptation. Bacteria in eco-cores were enumerated before and after exposure to PNP to determine whether the differences in mineralization rates correlated with changes in biomass. Samples were taken from the Escambia River in winter when the water temperature at the sampling site was 15°C; the cores were therefore incubated at 15°C during the experiment. Preexposure was done as described in Fig. 1. In the indicator core, the rate of mineralization increased abruptly after 110 h of incubation (Fig. 7). Preexposed and control cores respiked after 143 h showed the typical differences in rates of PNP mineralization. Thus, although the lag period was longer in the winter cores, adaptation patterns were similar to those of summer cores (Fig. 3).

Total numbers of heterotrophic bacteria and bacteria able to degrade PNP in pure culture were estimated by dilution and plating at various times during the experiment. The last three cores listed in Table 2 are those described in Fig. 7. The first three cores are duplicates that were not exposed to PNP. Total numbers of heterotrophic bacteria increased almost 10-fold in cores incubated for 143 h with no addition. Incubation for longer periods or in the presence of PNP led to some increases in the number of heterotrophs, but differences were slight. The number of PNP

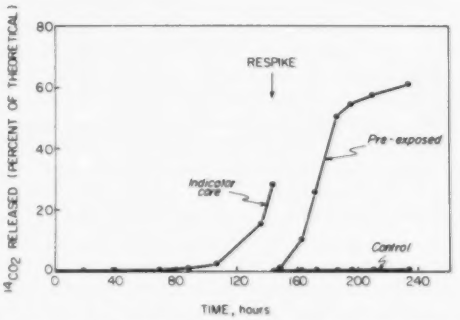

FIG. 7. *Mineralization of PNP in eco-cores taken from the Escambia River on 21 January 1980. The indicator was spiked initially with [^{14}C]PNP at a concentration of 1.08 μM, the preexposed core received an equal amount of unlabeled PNP, and the control core received no supplement. After 143 h of incubation at 15°C, the water was changed, and the preexposed and control cores were respiked with [^{14}C]PNP as described in the text. $^{14}CO_2$ release from the cores was followed during subsequent incubation.*

degraders increased 3 orders of magnitude in the core exposed to PNP for 143 h. There was an additional 10-fold increase in a similar core respiked with PNP and incubated 89 h longer. There was no detectable proliferation of PNP degraders in control cores.

The question remained whether the lack of an observable adaptation in cores taken from the salt marsh was due to a lower initial biomass in the samples than in the Escambia River samples. Therefore, core samples taken from the Escambia River were diluted 10-fold with filter-sterilized river water and used in a test for adaptation similar to that described in Fig. 1. Dilution had no discernable effect on the adaptation patterns. PNP mineralization in the preexposed core was much more rapid than in the control core (data not shown), which clearly indicated adaptation in the diluted system. Mineralization rates were similar to those obtained with undiluted cores

taken from the same site (Fig. 6). The results indicate that differences in adaptation patterns between Escambia River samples and Range Point samples were not due to differences in bacterial population size but were probably due to qualitative differences in the populations.

Concentration dependence. Preliminary experiments (Fig. 2) indicated that microbial populations in cores taken from the Escambia River would adapt to PNP concentrations as low as 0.45 μM. It seemed likely, however, that there would be a threshold concentration below which no adaptation could be detected. To define such a threshold, the concentration dependence of adaptation was investigated by the procedure used in earlier experiments (Fig. 1). Cores taken from the river site on 10 October 1979 were preexposed to various concentrations of unlabeled PNP; duplicate cores received no PNP. After incubation for 75 h, the water was changed, and radioactive PNP was added to the cores at concentrations equal to the respective preexposure concentrations. The preexposure period (before respike) was shorter than in the earlier experiments with Escambia River samples (Fig. 3). As a result, the differences in mineralization rates between preexposed and control cores were less drastic (Fig. 8).

Rates determined from the first three points in the curves shown in Fig. 8A and B were replotted as a function of initial PNP concentration (Fig. 8C). Rates of mineralization in the control cores were only slightly affected by PNP concentration in the range tested. In contrast, mineralization rates in preexposed cores showed a marked concentration dependence at concentrations above 0.29 μM. Rates of PNP mineralization in preexposed cores did not differ greatly from those in unexposed cores when both were supplemented with [^{14}C]PNP at concentrations below 0.43 μM. Cores preexposed to PNP at a concentration of 0.43 μM showed a somewhat higher (2.3-fold) mineralization rate than did the control cores. Preexposure to PNP at concentrations of 0.72 and 1.44 μM produced a much higher rate of mineralization of the labeled compound (5.3- and 8.5-fold, respectively).

TABLE 2. *PNP-dependent population changes in eco-cores*[a]

Core	PNP added at time (h)		Total incubation (h)	CFU/ml	
	0	143		Nutrient agar	PNP agar[b]
Initial	−	−	0	1.9×10^6	<10
Control	−	−	143	1.8×10^6	<10
Control	−	−	232	2.2×10^6	<10
Control	−	+	232	3.7×10^6	<10
Indicator	+	−	143	2.7×10^6	1.4×10^4
Preexposed	+	+	232	3.1×10^6	1.4×10^5

[a] Eco-cores were prepared and treated as described in the legend to Fig. 7. CFU, Colony-forming units.

[b] Only those colonies that caused decolorization of the PNP agar were counted.

DISCUSSION

We use the term "adaptation" to describe the phenomenon that causes a compound to be more rapidly degraded by the microbial population in a laboratory test system preexposed to the compound. Induction of catabolic enzyme synthesis probably takes place during adaptation, but we do not use the term "induction" because it is only one of the steps involved in adaptation.

When PNP was used as the test substrate, the rate of $^{14}CO_2$ release correlated well with the rate of disappearance of parent compound from the system. Changes in the rate of $^{14}CO_2$ release from radiolabeled organic compounds provided an excellent means of detecting adaptation in eco-cores. An abrupt increase in the rate of $^{14}CO_2$ release from systems spiked initially with radiolabeled PNP or MP indicated that the microbial population adapted to more rapidly degrade the parent compound. However, the lack of such an increase when low concentrations of test compound were used (Fig. 2) cannot be interpreted as lack of adaptation unless the appropriate respike experiments (Fig. 3) are performed. Determination of whether preexposed populations mineralized the test compound more rapidly than did similar unexposed populations is the only test that can be used to clearly demonstrate adaptation.

Unadapted eco-cores from both test sites showed low but detectable rates of PNP and MP mineralization. Such mineralization might be catalyzed by constitutive enzymes that are not specific for the substrates in question or catalyzed by specific enzymes present at low (uninduced) levels.

There are several possible explanations for the differences in adaptation between Range Point samples and Escambia River samples. (i) The bacterial biomass in the salt marsh samples may not have been high enough to allow adaptation. (ii) The higher salinity or lower concentrations of dissolved nutrients may have been limiting in salt marsh samples. (iii) Absence of specific organisms able to mineralize the test compounds in the salt marsh samples may have precluded adaptation.

Results obtained with diluted Escambia River samples indicate that differences in initial biomass at the two sites do not account for differences in adaptation patterns. Addition of yeast extract to shake flasks containing samples from Range Point and the test compounds did not

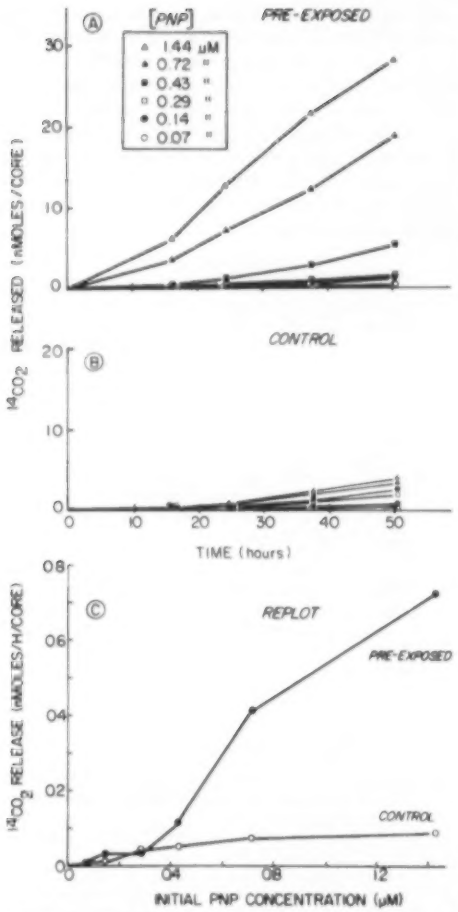

FIG. 8. *Concentration dependence. Eco-cores were taken on 10 October 1979 at the Escambia River site and spiked immediately with unlabeled PNP at the indicated concentration (A). Control cores (B) received no supplement. After incubation for 75 h, the water was removed and replaced with filtered river water, cores were respiked with [^{14}C]PNP at the indicated concentrations, and $^{14}CO_2$ release was followed during subsequent incubation. $^{14}CO_2$ evolution rates obtained from the first three points in (A) and (B) were plotted against initial PNP concentration in (C).*

facilitate adaptation; therefore, nutrient limitation was probably not the deciding factor. The addition of yeast extract to the flasks also increased the total biomass in the Range Point samples (data not shown) without influencing adaptation, which further suggests that total biomass does not determine whether adaptation will occur.

Differences in physical conditions at the two sites may have caused the differences in adaptation patterns. The most striking difference is in salinity, and there is some evidence to suggest that degradation rates of xenobiotics in saline environments differ from those in freshwater (3).

The third possible explanation, absence of specific organisms at Range Point, seems to be the most likely explanation for the differences in adaptation potential. Presence or absence of such organisms might be dictated by such physical conditions as salinity, by the history of the site (prior exposure), or by other factors. Our data indicate that organisms able to use PNP as a carbon source were present at the river site, but not at the salt marsh.

Organisms able to degrade MP in pure culture, however, were not isolated at either site. It is likely that degradation of MP is analogous to that of parathion, a two-step process involving an initial hydrolysis of the molecule and subsequent degradation of the PNP released (10). The hydrolytic cleavage and the catabolism of PNP may be carried out by different organisms, as in the system studied by Daughton and Hsieh (5). Such a system would explain our inability to isolate a single bacterium able to degrade MP, even though rapid mineralization occurred in mixed cultures from the Escambia River. Isolation of the organisms in pure culture with high concentrations of substrate does not prove that they were responsible for the observed mineralization at low concentrations in the eco-cores; likewise, failure to isolate such organisms from the Range Point samples does not prove that they were absent at the site. Our preliminary results suggest, however, that presence or absence of organisms with specific metabolic capabilities correlates with adaptation potential and, thus, the degradation rate of a particular compound at a particular site.

Other workers (2) have suggested that xenobiotic compounds may be persistent if introduced into the environment at low concentration. Our results indicate a threshold concentration in the range of 0.3 to 0.4 μM for the model compound PNP below which little or no adaptation is detectable. Some mineralization occurs at lower concentrations, but the rate is much lower than would be predicted by extrapolation of results obtained at higher concentrations. The ability to define an adaptation threshold may be very useful in predicting the fate of xenobiotics in a particular environment. If such an adaptation to degrade a particular compound can be expected in a given situation, then under most conditions the compound will disappear rapidly from the system. Therefore, it is crucial to fate assessment programs to determine if and when

adaptation occurs in natural environments.

Adaptation should not be considered an all-or-none phenomenon, but rather a quantitative one dependent on preexposure time and concentration. The degree of adaptation shown by populations in eco-cores varied from one experiment to the next. For example, cores preexposed for 4 days (Fig. 2) were adapted to a greater extent than those preexposed for 3 days (Fig. 6 and 8). The cores in the latter experiment were apparently respiked before adaptation was complete.

Our data indicate that preexposure increases the fraction of the population able to degrade PNP, which supports the idea that a particular segment of the population is selected by exposure to the nitrophenol. Addition of PNP to eco-cores did not greatly increase the total population of bacteria during the adaptation period. (The increase in the total number of bacteria in all cores was apparently due to the "bottle effect" [12]). There was, however, a disproportionate increase in the fraction of the population able to degrade PNP. Such an increase could easily account for the observed increases in mineralization rate.

Baughman et al. (1) have shown that for certain compounds the rate of biodegradation in mixed culture systems is determined by the structure of the compound and by the total biomass in the system. Our results indicate that the ability of the microbial population to adapt to rapidly degrade a compound may, in some cases, be an additional rate-determining factor. The data suggest that to predict the rate of biodegradation in a particular environment, it is important to determine whether the population in that environment will adapt to rapidly degrade the xenobiotic in question, particularly if degradation of the compound requires specific reactions such as removal of a nitro group or attack on an aromatic ring. In such cases, measurement of the total biomass may not be sufficient to predict biodegradation rates. The probable residence time of a pollutant in a particular environment must also be considered, since adaptation is time dependent. In situations in which exposure is chronic, the rate of biodegradation might be very rapid compared with that in areas receiving intermittent exposure.

We do not understand what factors determine whether microbial populations will adapt to more rapidly degrade a particular organic compound; however, our results show that adaptation can occur in one environment but not in another. Moreover, it may occur with one compound and not with another in the same environment. Therefore, the effects of adaptation must be carefully considered before the results of laboratory biodegradation experiments can be extrapolated to predict biodegradation rates in natural environments.

LITERATURE CITED

1. **Baughman, G. L., D. F. Paris, and W. C. Steen.** 1980. Quantitative expression of biotransformation rate, p. 105–111. *In* A. W. Maki, K. L. Dickson, and J. Cairns, Jr. (ed.), Biotransformation and fate of chemicals in the aquatic environment. American Society for Microbiology, Washington, D.C.
2. **Boethling, R. S., and M. Alexander.** 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. **37**:1211–1216.
3. **Bourquin, A. W., and V. A. Przybyszewski.** 1977. Distribution of bacteria with nitrilotriacetate-degrading potential in an estuarine environment. Appl. Environ. Microbiol. **34**:411–418.
4. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.
5. **Daughton, C. G., and D. Hsieh.** 1977. Parathion utilization by bacterial symbionts in a chemostat. Appl. Environ. Microbiol. **34**:175–184.
6. **Haller, H. D.** 1978. Degradation of mono-substituted benzoates and phenols by wastewater. J. Water Pollut. Control Fed. **50**:2771–2777.
7. **Hollibaugh, J. T.** 1979. Metabolic adaptation in natural bacterial populations supplemented with selected amino acids. Estuarine Coastal Mar. Sci. **9**:215–230.
8. **Larson, R. J.** 1979. Estimation of biodegradation potential of xenobiotic organic chemicals. Appl. Environ. Microbiol. **38**:1153–1161.
9. **Pritchard, P. H., A. W. Bourquin, H. L. Fredrickson, and T. Maziarz.** 1979. System design factors affecting environmental fate studies in microcosms, p. 251–272. *In* A. W. Bourquin and P. H. Pritchard (ed.), Microbial degradation of pollutants in marine environments: proceedings of the workshop. U.S. Environmental Protection Agency, EPA-600/9-79-012. Washington, D.C.
10. **Siddaramappa, R., K. P. Rajaram, and N. Sethunathan.** 1973. Degradation of parathion by bacteria isolated from flooded soil. Appl. Environ. Microbiol. **26**: 846–849.
11. **Skerman, V. B. D.** 1967. A guide to the identification of the genera of bacteria. The Williams and Wilkins Co., Baltimore.
12. **Wright, R. T.** 1979. Natural heterotrophic activity in estuarine and coastal waters, p. 119–134. *In* A. W. Bourquin and P. H. Pritchard (ed.), Microbial degradation of pollutants in marine environments: proceedings of the workshop. U.S. Environmental Protection Agency, EPA-600/9-79-012, Washington, D.C.